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U.S. ARMY MEDICAL RESEARCH
INSTITUTE OF CHEMICAL DEFENSE

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CARDIAC PERFUSION VS. IMMERSION FIXATION FOR THE PRODUCTION OF ARTIFACT-FREE BRAIN SECTIONS

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The postmortem chemical fixation of brain routinely results in some degree of artifactual tissue changes which are difficult to differentiate from preexisting pathology. More specifically, by light microscopy of hematoxylin and eosin-stained sections, hyperchromatic, purple neurons are frequently seen in completely normal brains. Preexisting early neuronal degeneration, however, may result in similar staining characteristics. Thus, a fixation technique which minimizes the occurrence of these dark neurons and other artifacts would be beneficial in the interpretation of subtle brain pathology. This study was conducted to evaluate and compare several methods of tissue fixation to determine which technique produced the most artifact-free brain tissues. Rats in 9 groups of 10 each were euthanatized and their brains immediately fixed by intravascular perfusion or immersion in neutral buffered formalin (NBF) or Karnovsky's in Locke's buffer (KLB). Procedural variables also included a pre-KLB fixation flush with heparinized saline and post-fixation buffer wash, clamping of the descending aorta

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during the procedure, varying perfusion pressures, and different anesthetics. All tissues were routinely paraffin embedded, cut at five microns, H&E stained, and examined by a panel of veterinary pathologists. Slides were blind coded and scored individually by pathologists for occurrence of artifacts. The data indicated that perfusion fixation in rats with 10% NBF alone significantly decreased the occurrence of artifactual hyperchromatic neurons over that produced by immersion fixation for light microscopy. Brain studies involving ultrastructural examinations would be better accommodated by perfusion fixation with KLB preceded by a heparinized saline flush, which also produces acceptable results at the light microscopic level.

PREFACE

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INTRODUCTION

The pathological examination of central nervous system tissue is crucial to the U.S. Army Medical Research Institute of Chemical Defense's (USAMRICD) effort to evaluate nerve agent toxicity. The pathology of experimental nerve agent poisoning has been described (McLeod and Wall, 1987), but ongoing research with nerve agents and prophylactic and therapeutic (P&T) compounds requires continued histopathological examination in laboratory animal models, principally rodents. Chemical (commonly aldehyde) fixation of animal tissues is required before subsequent processing for examination by a pathologist (Baker, 1958). Proper tissue handling and methods of fixation are important in producing a high quality specimen for microscopic study, because tissue fixation itself induces artifacts, and many additional artifacts can be caused by multiple procedural factors (Thompson and Luna, 1978). Some of these tissue fixation artifacts are difficult to differentiate from subtle pathological changes. For example, by light microscopy of hematoxylin and eosin (H&E)-stained brain sections, purple, "dark" neurons are frequently seen in completely normal brains. However, preexisting early neuronal degeneration may result in similar staining characteristics. Thus, a fixation technique which minimizes the occurrence of these dark neurons and other artifacts would be beneficial in the interpretation of subtle brain pathology.

Immersion in 10% neutral buffered formalin (or other aldehyde fixative) is probably the most common form of tissue fixation. This is simple, compatible with most tissue stains, and affords relatively rapid penetration of tissues. Good fixation is still dependent upon complete and rapid penetration into the tissues because cells and tissues begin autolysis immediately following death, or even under significant hypoxia. This means tissues should be dissected immediately and generally be no larger than two to five millimeters thick. For tissues amenable to fresh slicing this presents no difficulty, but the brain, due to its soft nature, cannot be easily sliced before fixation without inducing many artifacts. Aldehyde fixatives can diffuse to the center of the brain in a few days, but the brain can shrink as much as 30%. Intuitively then, immersion fixation of larger brains (dogs, monkeys, etc.) would be slower and less suitable than in smaller brains (rats, mice).

An alternative to immersion fixation is intravascular perfusion of the selected fixative. This allows perfuse, immediate contact of the fixative with tissues at the capillary level, and is considered essential for some ultrastructural studies, such as those targeting vascular or central nervous system tissues. Perfusion-fixed tissues are usually post-fixed by immersion in the same fixative. Most comparative studies have applauded the results of perfusion fixation over immersion techniques. Gertz et al. (1975), utilizing cardiac perfusion in a study of vascular endothelium, concluded that perfusion produces less cellular distortion and disruption than do immersion-fixed tissues. In perfusion-fixed brain specimens, Johnson et al. (1978) found that the meninges were closely adherent to the molecular layer of the cerebellum, and perivascular spaces and dark neurons,

which are common artifacts in immersion-fixed tissues, were rarely seen. Additionally, Purkinje cells and the neurons in the medullary nuclei were uniform in staining characteristics.

All perfusion techniques are not identical. Some of the variables in the techniques include choice of the fixative, how the fixative is administered (i.e., gravity flow, peristaltic pump, hand syringe), where vascular cannalization is achieved (i.e., cardiac, aortic), time length of perfusion, site of exsanguination (i.e., right auricle, jugular vein, femoral vein), whole body versus upper or lower body perfusion, and option of prefixative perfusion (saline, heparin). Stickrod and Stansifer (1981) used a small 60 RPM mini-peristaltic pump to administer their perfusates, and felt the pump had an advantage over gravitational force, in which fluctuations occur in pressure as the fluid levels in the reservoir drop. Cummings and Petras (1977) reported euthanatizing neonatal dogs with intravenous heparinized sodium pentobarbital before cardiac perfusion with physiological saline or McEwen's balanced salt solution, followed by their choice of fixative. For brain perfusion several investigators have described clamping off the descending aorta before perfusion begins (Bondonna et al., 1977; Stickrod and Stansifer, 1981; and Kalimo et al., 1981). Most of these investigators also utilized a saline flush to remove the blood from the vasculature before the fixative is infused; however, Bondonna found that a saline flush was unnecessary when perfusing with 50% formalin followed by a 10% sucrose-formalin solution. They also found that more rapid perfusion of fixative was better in clearing the brain of all traces of blood than perfusing first with saline.

Here at the USAMRICD the Comparative Pathology Branch regularly examines the brains of rats exposed to nerve agents or P&T compounds for potential deleterious effects. Thus, a rapid, optimal method of tissue fixation which minimized artifacts, such as purple neurons, was needed. This study was conducted to evaluate and compare several methods of tissue fixation to determine which technique produced the most artifact-free brain tissues.

OBJECTIVES

The purpose of this experiment was to develop a standardized method for fixation of brain tissue, resulting in artifact-free specimens.

The effects of cardiac perfusion and immersion fixation were compared, using 10% neutral buffered formalin (NBF) and 1/2 strength Karnovsky's in Locke's buffer (KLB) as fixatives. The following questions were addressed:

1. Is a flush with heparinized saline necessary before perfusion with Karnovsky's fixative?

2. Is clamping the descending aorta effective in producing higher quality specimens?

3. Which fixative is better for producing artifact-free specimens at the light microscopic level?

4. Are steady perfusion pressures necessary to ensure good fixation?

5. Do different anesthetics produce a difference in specimen quality?

MATERIALS AND METHODS

Experimental Design and General Procedures

A total of 119 male Crl:CD® BR VAF/Plus® rats Rattus norvegicus, 180-220g, were used. These animals were quarantined upon arrival and screened for evidence of disease before use. They were maintained under an AAALAC accredited animal care and use program in plastic cages (Lab Products, Inc., Maywood NJ). The rats were housed in groups of three (3), on ground corncob contact bedding (Bed O'Cobs; Andersons COB Division, Maumee, OH) that was changed two times each week. They were provided commercial certified rodent ration (Zeigler Bros., Inc., Gardners, PA) and tap water ad libitum. Animal holding rooms were maintained at 21° ± 2° C with 50% ± 10% relative humidity using at least 10 complete air changes per hour of 100% conditioned fresh air. All animals were on a twelve-hour light/dark full spectrum lighting cycle with no twilight.

Rats were used as the animal of choice because they are commonly used for OP studies in the Institute, and the brain histology is well defined. They are of sufficient size to manipulate, and uniform weights are readily available so fewer animals were needed to eliminate size variables. The experiment consisted of three parts.

Part 1. A preliminary experiment was performed using five rats in an attempt to measure actual carotid blood pressure during perfusion, and to correlate that pressure to the pressure seen on the perfusion apparatus' pressure gauge. Five rats were used as follows:

Rat #1 - perfusion with saline followed by NBF, with perfusion pressures varying as high as 160 mm Hg.

Rat #2 - same as rat #1, with pressures up to 300 mm Hg.

Rat #3 - perfusion with NBF only, varying pressures, descending aorta clamped prior to perfusion.

Rat #4 - same as rat #3, but with a steady rate of perfusion (120 mm Hg).

Rat #5 - same as rat #4, but without clamping of the descending aorta.

Part 2. The main experiment consisted of nine groups of rats, 10 rats per group, as outlined below:

Group 1 - perfusion with 50 cc intracardiac (left ventricle) 10% NBF at 120 mm Hg.[1]

Group 2 - perfusion with 25 cc heparinized saline i.c. followed by 50 cc 1/2 strength KLB[2] at 120 mm Hg. Heparin dose was 250 I.U./kg.

Group 3 - perfusion with 50 cc 1/2 strength KLB at 120 mm Hg.

Group 4 - perfusion with 50 cc 10% NFB at 120 mm Hg, with descending aorta clamped.

Group 5 - immersion fixation of brains in 10% NBF.

Group 6 - immersion fixation of brains in 1/2 strength KLB.

Group 7 - same as Group 6, followed by a buffer wash prior to histoprocessing.

Group 8 - same as Group 2, followed by a buffer wash.

Group 9 - same as Group 3, followed by a buffer wash.

Part 3. A follow-up experiment was conducted to compare the quality of brain sections from rats given different anesthetics prior to perfusion or immersion fixation. Two rat brains were perfusion fixed and two were immersion fixed after sodium pentobarbital, Halothane® and Forane® anesthesia. Two rats had their brains immersion fixed after T-61® and CO_2 euthanasia (no perfusions). In addition, the brains of eight animals from an older study which had been perfused with quantities of 10% NBF in excess of 50 ml were compared to those brains from Groups 1 and 5 of the main

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[1]Pressure chosen is based on the normal mean arterial pressure of an adult male Charles River SD rat of 125 $\pm$ 10.9 mm Hg.

[2]This fixative can be used for electron microscopy as well as light microscopy. Osmolarity of the buffer is 313 mOsm, in the range of the osmolarity of rat plasma (228-336 mOsm). Fixative was chilled at 4° C.

experiment. This comparison was made to ascertain if 50 ml of the fixative is a sufficient quantity for total fixation.

Technical Methods

Part 1. Five rats were deeply anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally). Their right carotid arteries were cannulated with pressure sensitive probes and connected to a Gould Physiograph to record arterial blood pressure. A connection was also made between the perfusion syringe with pressure gauge and the Gould Physiograph. The animals were then perfused (by the method described in Part 2, below) with either 50 ml lactated Ringer's solution with dextrose followed by 50 ml 10% NBF, or 10% NBF alone. After an initial baseline recording of the animals' blood pressure, recorded tracings were made of carotid and syringe pressures for the duration of the perfusions.

Part 2. Rats were hand held and anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally), placed on a cork board, and pinned down in dorsal recumbency. The sternum was reflected upwards and the heart exposed. The tip of an 18-gauge 1-1/2" needle attached to a 50-cc perfusion syringe was inserted into the left ventricle of the animal, and a small incision made in the right ventricle. For those animals that received saline flushes, a 25-cc dose of heparinized saline was injected, after which the syringe was removed leaving the needle in the left ventricle and replaced with a syringe containing fixative. The descending aorta was clamped with a hemostat prior to NBF infusion for the animals in Group 4. All perfusions were conducted at a steady pressure of 120 mm Hg, as indicated by the pressure gauge attached to the perfusion syringe. Animals who were not perfused (Groups 5 and 6) had their sternums reflected and were exanguinated via a slit in the descending aorta. All brains were removed and placed in 50 cc of the appropriate fixative for additional immersion fixation. After 48 hours, a coronal section was taken through the center of the cerebrum and the hippocampus. After 24 more hours in fresh fixative (and then a buffer wash for those animals in Groups 7-9), the sections were routinely processed in paraffin and stained with hematoxylin and eosin.

Part 3. The rats were either anesthetized with 40 mg/kg sodium pentobarbital i.p., euthanatized with 5 ml/kg T-61® i.p., asphyxiated in a CO_2 chamber, or placed in an anesthetic jar with Halothane®-saturated or Forane®-saturated gauze until a deep plane of surgical anesthesia was reached. The rats were separated from the anesthetic-soaked gauze by a metal platform, so no direct contact causing possible eye irritation was possible. Either rats were perfused with formalin only (> 50 ml, until perfusion was judged to be adequate based on body rigidity) or their brains were removed after exanguination and immersion fixed. All brains were processed for microscopic evaluation as described in Part 2.

Data Analysis

Part 1. Graphs were prepared to plot carotid pressure and syringe pressure vs. time, for the purpose of visually correlating the two pressures. Cardiovascular effects from clamping the descending aortas in two of the rats prior to perfusion with the fixative were also examined using these graphs. The accuracy of the gauge on the perfusion syringe was verified by comparing the sighted pressures from the gauge during the procedure to those recorded by the Physiograph.

Part 2. Brain slides were labelled using a keyed table of random numbers and submitted to veterinary pathologists for examination without knowledge of the method of fixation. Identical fields were examined by all pathologists. Brains were examined for the presence or absence of perineural spaces, perivascular spaces, dark cells, hemorrhage, thrombi and vascular endothelial damage. The following rating system was used:

1. Neurons:

   (6) Dark cells not observed, or only very rarely seen.

   (4) A few dark cells, with occasional small clusters of dark cells seen.

   (2) Mild numbers of dark cells, with occasional large clusters seen.

   (0) Many dark cells with frequent large clusters of dark cells.

2. Perineuronal/perivascular spaces:

   (2) Essentially no (or rare) perineuronal/perivascular spaces found.

   (1) Occasional spaces observed of only mild severity.

   (0) Numerous spaces observed and/or marked severity.

3. Red Blood Cells (RBCs):

   (2) Essentially no (or rare) RBCs in vasculature.

   (1) Mild numbers of RBCs in many areas.

   (0) Vasculature packed with numerous RBCs.

This rating system emphasized the importance of good fixation of the neurons, but also took into account the less critical artifact due to shrinkage. Incomplete flushing of the vasculature based on the presence of RBCs was also taken into consideration. Scores from each of the three rating categories were added up, for a maximum possible overall score of 10. Kruskal-Wallis (nonparametric) tests were run on the group means to test for significant differences, using the average total score of all pathologists for each animal.

Part 3. All brains were rated by one pathologist according to the same criteria used in Part 2. Since the number of animals tested was not high enough for valid statistical analysis, a subjective opinion was rendered as to which anesthetic/fixation technique produced the overall best result. The quantity of fixative necessary for adequate fixation was also judged subjectively by the same pathologist in the comparison with older study animals. These older animals had been perfused via gravitation force with amounts of fixative greatly in excess of the 50 cc used in Part 2.

RESULTS AND DISCUSSION

The preliminary study to correlate carotid pressure with syringe pressure provided some interesting results. Although there were not enough animals tested to provide valid statistical analyses, there are some general conclusions that can be made:

1. Varying pressures with formalin (large fluctuations) result in an initial response followed by a steady drop off in carotid pressure (Figures 1-3). Steady pressures with formalin result in steady carotid pressures (Figures 4-5).

2. No matter how high the perfusion pressure gets (even up to 300 mm Hg), carotid pressure remains below 50% of the animal's normal baseline pressure (Figures 1-5).

3. Clamping the descending aorta just prior to perfusion does not substantially increase the carotid pressure (Figures 3-4).

These results indicate that, although steady perfusion rates are desirable, perfusing at 120 mm Hg (as is commonly done for rodents) does not mean the carotid and cerebral arteries are exhibiting that same pressure. Opening the thoracic cavity to perform the perfusion technique drastically compromises cardiovascular integrity. Typically, rodents are perfused this way and not via cannulation of the abdominal aorta (without opening the thoracic cavity) as is sometimes performed in larger animal species. Cannulation of the abdominal aorta in rats proves to be very tedious and time consuming.

Rank ordering of group means in the main experiment (using average scores of all pathologists) resulted in the following order of preference for fixation techniques: formalin perfusion groups, saline/Karnovsky's perfusion groups, formalin immersion, Karnovsky's immersion, and Karnovsky's perfusion (no pre-fixative saline flush) groups. Kruskal-Wallis tests run on the group means revealed some statistically significant differences among the groups ($p<0.05$, Table 1). There was no difference between immersion in either fixative. Clamping the descending aorta made no difference. There was no difference between the saline/Karnovsky's groups with respect to washing with buffer prior to histological processing. There were differences, however, between perfusing with saline/Karnovsky's and just Karnovsky's, and between perfusing with NBF and Karnovsky's with no preceding saline flush. These differences indicate that when perfusing with Karnovsky's, it is advisable to precede with a saline flush. A previous study conducted by other investigators has shown that saline flushes are not necessary with rapid perfusion of some formaldehyde-based fixatives in small rodents (Bondonna et al., 1977). At the same time, saline flushes are necessary in larger animal species to prevent clotting in the microvasculature of the brain with subsequent blocking of the flow of fixative.

There were also differences between perfusing with NBF or saline/Karnovsky's and immersion fixation with either fixative. This supports the generally accepted belief that better fixation can be attained with a perfusion fixation technique. There was no significant difference between perfusing with 10% NBF or saline/Karnovsky's at the light microscopic level.

In the final phase of this experiment, various anesthetics were examined to determine which, if any, produced the best overall histological results. None of the methods of euthanasia were markedly superior to any of the others, although overall the sodium pentobarbital tended to produce somewhat fewer dark neurons than did the other drugs. Perfusion did not visually appear to produce significantly better results than immersion fixation for purposes of light microscopy; however, there was some question as to whether the perfusion was adequately performed since some RBCs and perivascular spaces were frequently present. Perfusing with amounts of NBF greater than 50 cc via gravity perfusion appeared to produce better results than perfusing with just 50 cc.

CONCLUSIONS AND RECOMMENDATIONS

In laboratory rats, perfusion with 10% neutral buffered formalin produced the most artifact-free specimens. This method of tissue fixation should be utilized if neural studies are being conducted in which interpretation of subtle brain pathology is required. Fixation may be improved and the occurrence of artifacts reduced if the brain is further immersed in fixative for 2-3 days in situ before removal from the cranium. The point of delaying dissection for more than 24 hours following perfusion is supported by the findings of Cammermeyer (1978), who concluded that the dark neurons normally seen

in immersion-fixed brain tissue can be induced by postmortem trauma occurring when saline perfusion was delayed or slow; the amount of saline was too great, allowing the tissues to swell; the amount of perfusate was inadequate; or the necropsy was performed shortly after the perfusion. Cammermeyer postulated that as a consequence of regional differences in intensity and speed of fixation, distortion during extraction of the brain may activate a stress force in the transitional zones where incompletely fixed neurons become affected and acquire an abnormal affinity for aniline dyes and silver (hence appear "dark").

Sodium pentobarbital (40 mg/kg i.p.) is an acceptable anesthetic and tends to produce fewer dark neurons than the other anesthetics tested.

Gravity perfusion should be sufficient for steady perfusion pressures. The very small fluctuations that may occur due to dropping fluid levels in the reservoir should not have any large effects on the carotid pressures during the perfusion procedure. Clamping of the descending aorta prior to perfusion of the fixative is not necessary in rats. Quantities of fixative in excess of 50 cc are recommended for the best results.

Studies where electron microscopic examination is anticipated would be better served by perfusing with an EM fixative such as 1/2 strength Karnovsky's in Locke's buffer, which produces comparable results with 10% neutral buffered formalin at the light microscopic level. This EM fixative needs to be proceeded by a heparinized saline flush. Buffer washes just prior to histoprocessing are not necessary for light microscopic evaluation.

For larger animal species, which have relatively larger brains (hence further distances for the fixative to traverse by immersion fixation), it is highly recommended that perfusion fixation be utilized, with heparinized saline being used for both light and EM fixatives. A pre-fixative flush of saline is necessary to flush out the large blood volume and assure that the deep micro- vasculature is not clotted so that the fixative can reach the deep areas. However, the question arises as to whether hypoxic changes in the brain are occurring during the saline perfusion, before the fixative has been introduced. It is suspected that hypoxia is indeed being induced during the extended saline profusion typically required to flush the blood volume in large animals. These hypoxic changes, if they occur, may make diagnosing soman-exposed brains difficult since these often display hypoxic-like changes on their own. This possible problem might bear further investigation. However, immersion fixation in these species may result in depth-related variations in staining intensity (Beach et al., 1987) and improperly fixed areas.

FIGURE 1

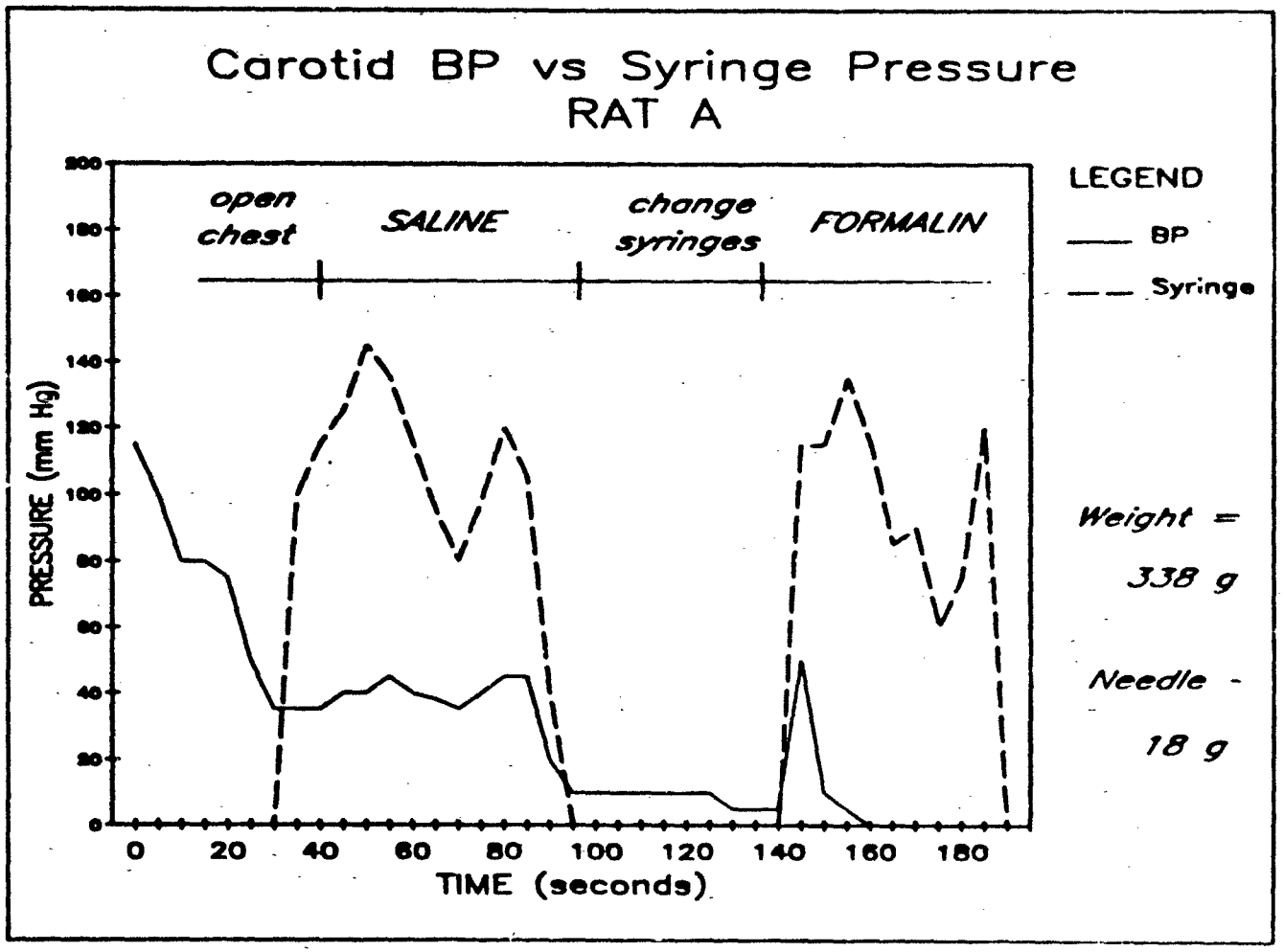
Carotid BP vs Syringe Pressure
RAT A
open chest
SALINE
change syringes
FORMALIN
LEGEND
BP
Syringe
PRESSURE (mm Hg)
TIME (seconds)
0
20
40
60
80
100
120
140
160
180
Weight = 338 g
Needle - 18 g

FIGURE 2

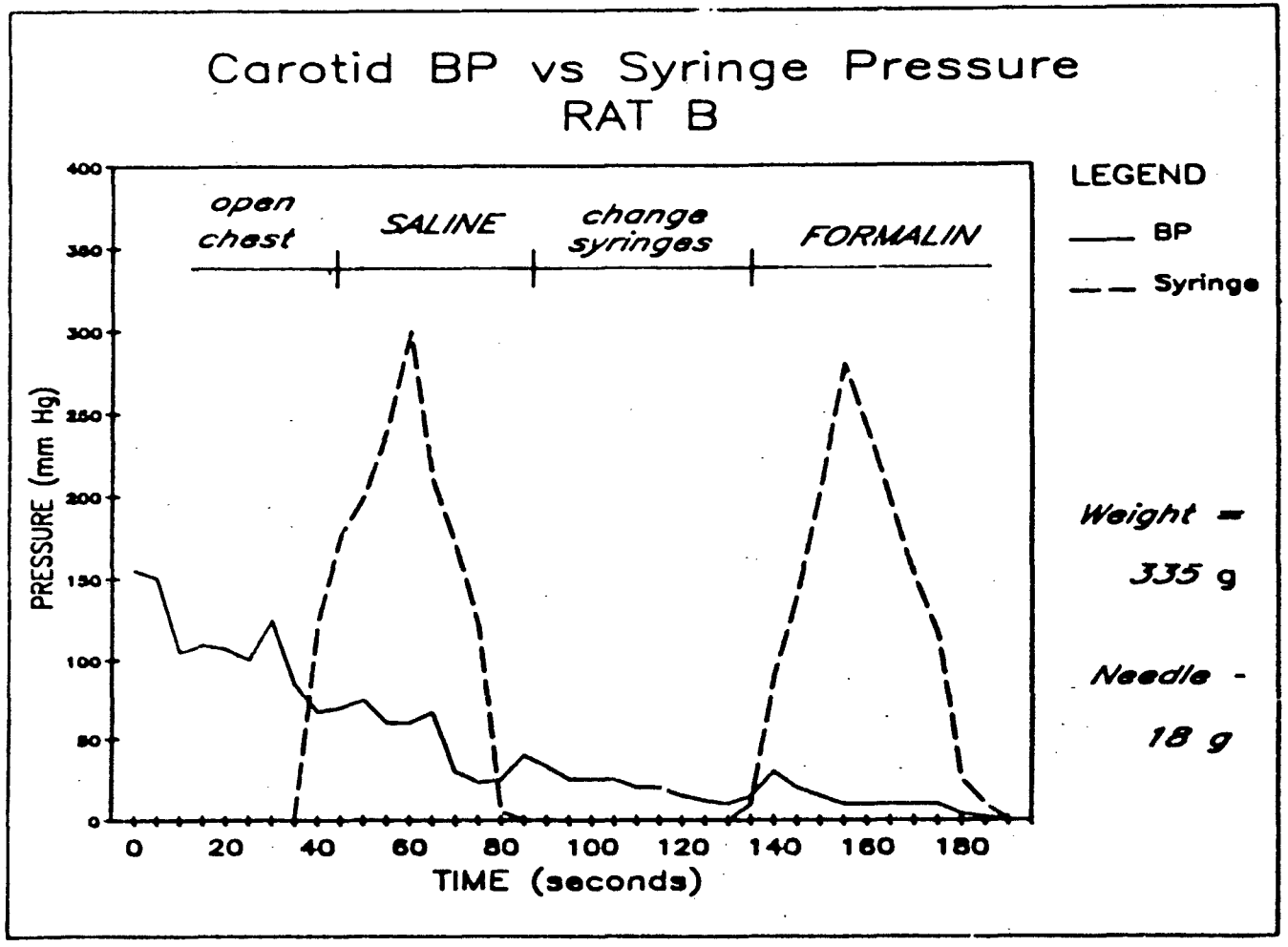
Carotid BP vs Syringe Pressure
RAT B
open chest
SALINE
change syringes
FORMALIN
LEGEND
BP
Syringe
Weight = 335 g
Needle - 18 g
PRESSURE (mm Hg)
TIME (seconds)
0
20
40
60
80
100
120
140
160
180
400
350
300
250
200
150
100
50
0

FIGURE 3

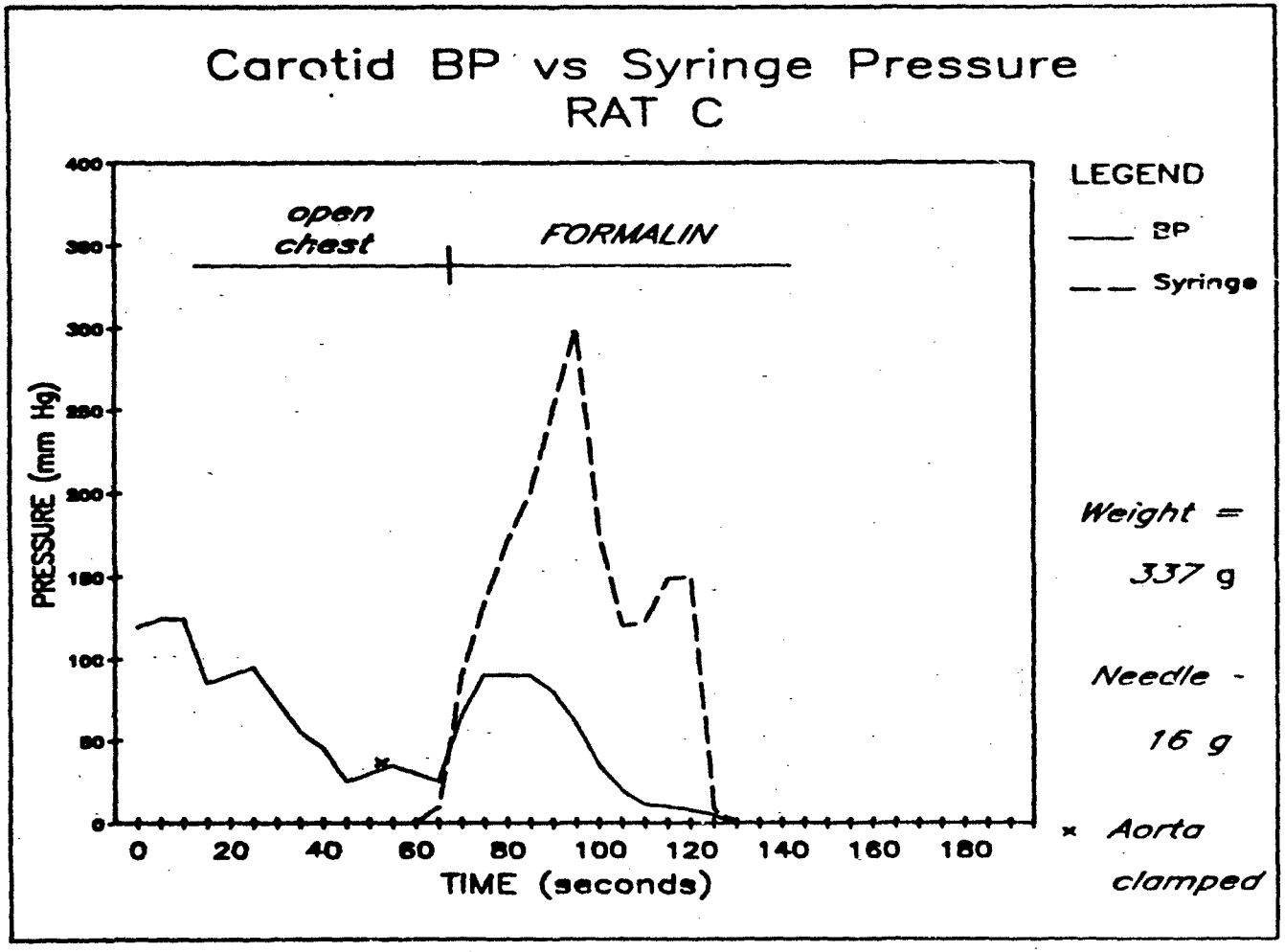
Carotid BP vs Syringe Pressure
RAT C
open chest
FORMALIN
PRESSURE (mm Hg)
400
300
200
100
0
0 20 40 60 80 100 120 140 160 180
TIME (seconds)
LEGEND
BP
Syringe
Weight = 337 g
Needle - 16 g
× Aorta clamped

FIGURE 4

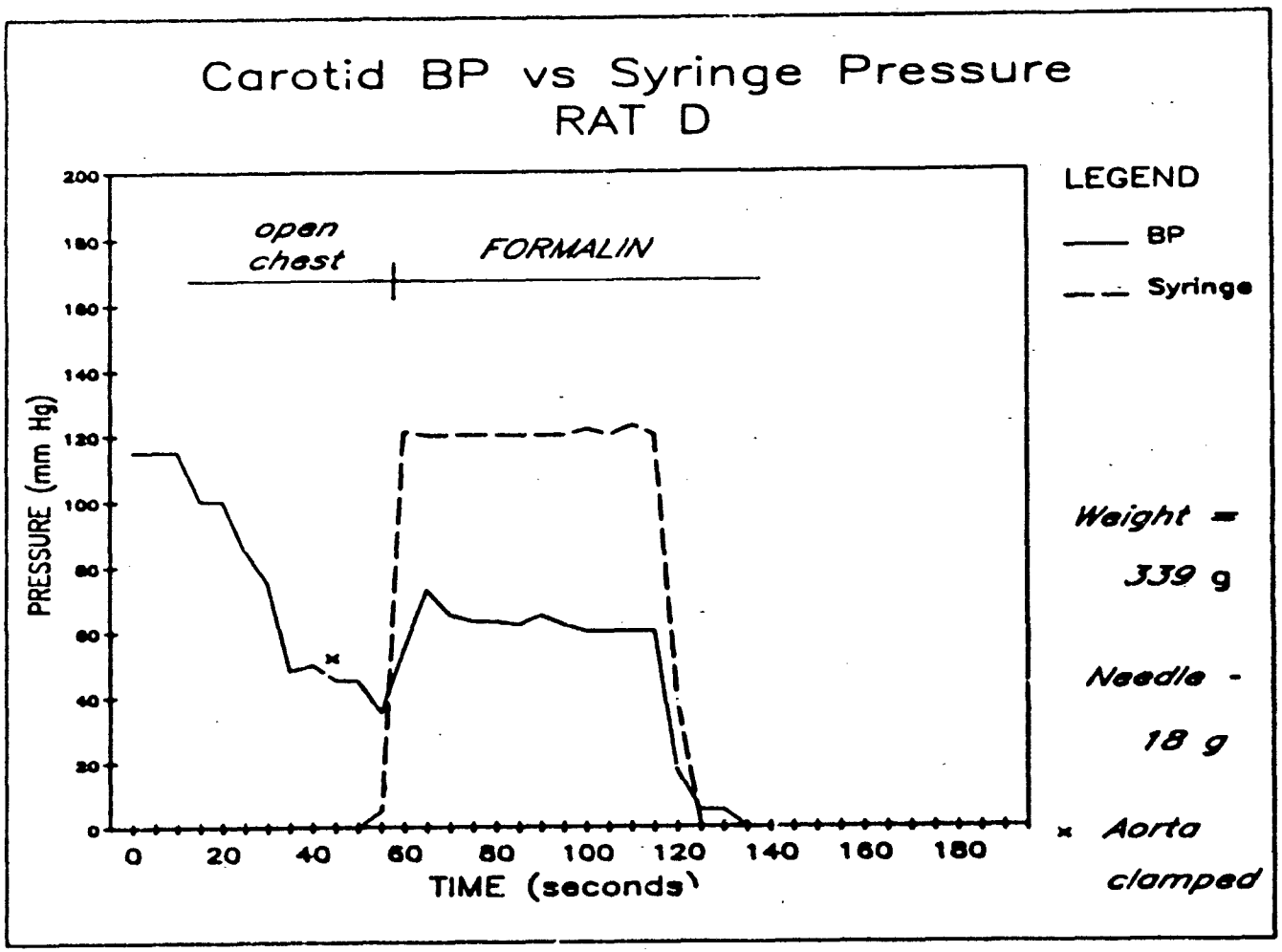
Carotid BP vs Syringe Pressure
RAT D
open chest
FORMALIN
PRESSURE (mm Hg)
TIME (seconds)
LEGEND
BP
Syringe
Weight = 339 g
Needle - 18 g
× Aorta clamped

FIGURE 5

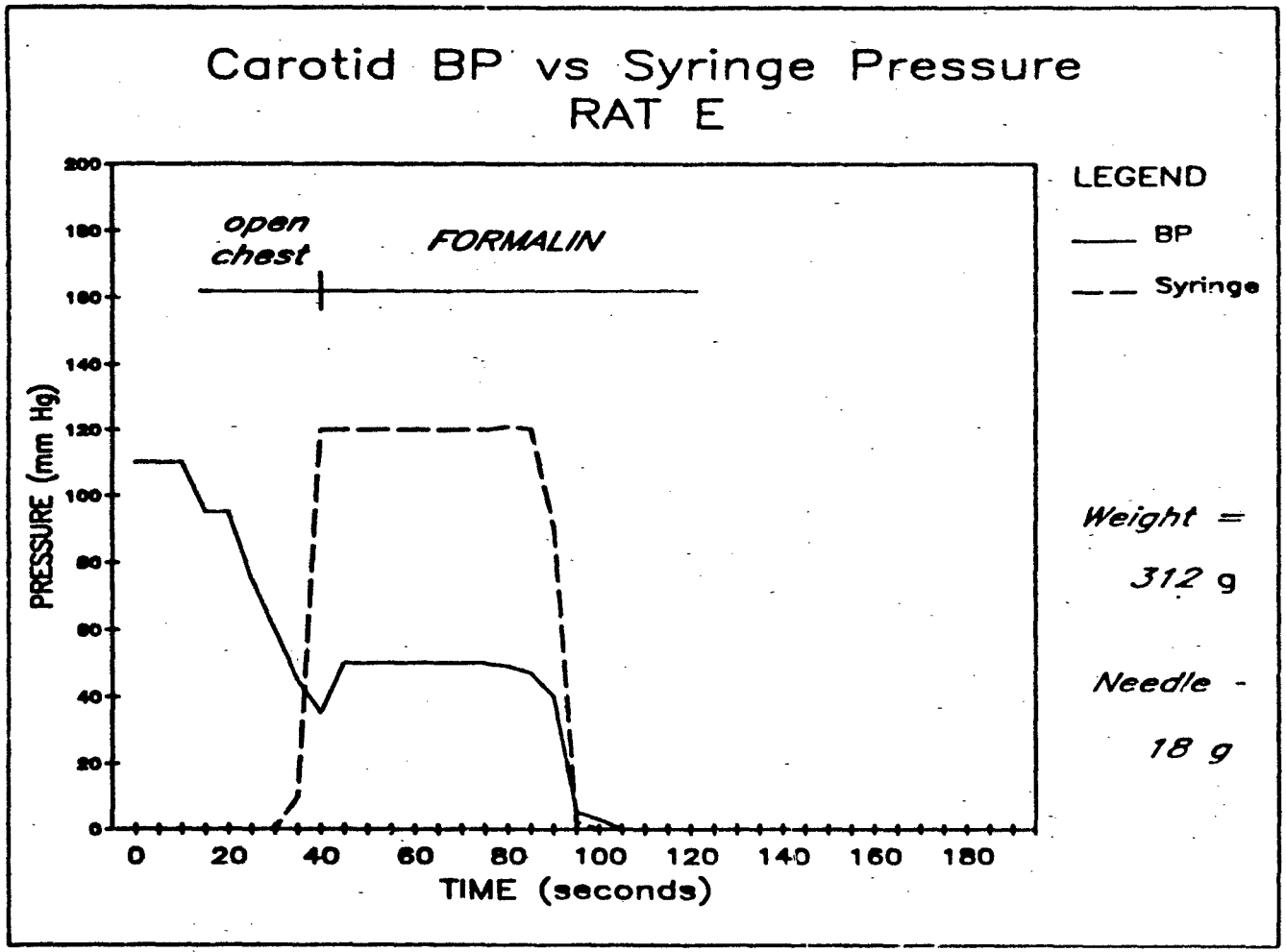
Carotid BP vs Syringe Pressure
RAT E
open chest
FORMALIN
LEGEND
BP
Syringe
Weight = 312 g
Needle - 18 g
PRESSURE (mm Hg)
TIME (seconds)
0
20
40
60
80
100
120
140
160
180
200

TABLE 1

Cardiac Perfusion Versus Immersion Fixation
Protocol # 1-21-83-017-A-206

Kruskal-Wallis Tests on Group Means
(using average scores of all pathologists)

COMPARISON	p value
G1 vs G3	.0013
G1 vs G5	.0058
G1 vs G6	.0091
G1 vs G7	.0022
G1 vs G8	.0312
G1 vs G9	.0032
G2 vs G3	.0009
G2 vs G5	.0211
G2 vs G6	.0173
G2 vs G7	.0284
G2 vs G9	.0036
G3 vs G4	.0041
G3 vs G5	.0494
G3 vs G8	.0041
G4 vs G6	.0376
G4 vs G9	.0233
G8 vs G9	.0233

Treatment Groups

Group 1 - perfusion with 50 cc intracardiac (left ventricle) 10% neutral buffered formalin (NBF) at 120 mm Hg.

Group 2 - perfusion with 25 cc heparinized saline i.c. followed by 50 cc 1/2 strength Karnovsky's in Locke's Buffer (KLB) at 120 mm Hg.

Group 3 - perfusion with 50 cc 1/2 strength KLB at 120 mm Hg.

Group 4 - perfusion with 50cc 10% NBF at 120 mm Hg, with descending aorta clamped.

Group 5 - Immersion fixation of brains in 10% NBF.

Group 6 - Immersion fixation of brains in 1/2 strength KLB.

Group 7 - same as Group 6, followed by a buffer wash prior to histoprocessing

Group 8 - same as Group 2, followed by a buffer wash

Group 9 - same as Group 3, followed by a buffer wash

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